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# New Method for Purifying *Coxiella burnetii* by Dextran Sulfate Precipitation Followed by Centrifugation

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ABSTRACT

DAVIS, J. L. (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), AND W. C. PATRICK, III. New method for purifying *Coxiella burnetii* by dextran sulfate precipitation followed by centrifugation. Appl. Microbiol. 13:99-102. 1965.—This paper describes a simple method for purifying *Coxiella burnetii* cultivated in the chick embryo. The procedure is based upon the preliminary clarification of infected whole-egg suspensions by precipitating the low-density lipoproteins and other extraneous solids with dextran sulfate, calcium chloride, and sodium chloride solutions, and then concentrating the rickettsiae by centrifugation.

Methods for purifying and concentrating the rickettsiae, and more specifically, *Coxiella burnetii*, have been described. A limited number of these methods are cited to illustrate the diversity of procedures employed: use of ether (Craigie, 1945), use of Celite (Shepard and Topping, 1947), differential centrifugation (Bovarnick and Snyder, 1949), use of bentonite (Pologov and Yakovlev, 1961), density-gradient studies (Ribi and Hoyer, 1960), differential centrifugation from 1 M KCl followed by dimethyl-sulfoxide and dimethylacetamide (Ormsbee, Bell, and Lackman, 1962).

Although there are many procedures for purifying *C. burnetii* from the tissue of the chick embryo, methods for efficiently separating the organism from the lipoprotein components and at the same time maintaining viability are limited. The extraction of lipids with ethyl ether (Ormsbee, 1962) or other nonaqueous organic solvents yields purified egg suspensions and reduced rickettsial viability. Sakagami and Zilversmit (1962) demonstrated the feasibility of dextran sulfate-calcium chloride-sodium chloride solutions to separate high- and low-density lipoproteins from dog serum when centrifuged at 4,000 × *g* for 10 min. They designated the supernatant fraction as dextran sulfate-soluble lipoprotein of high density (1.063 to 1.210), and the precipitate as dextran sulfate-precipitable lipoprotein of low density (1.063 or less).

The successful isolation of lipoproteins in dog serum by dextran sulfate suggested the adaptation of this technique to the purification of *C. burnetii* from the chick-embryo substrate. This communication describes the simple procedure developed for purifying *C. burnetii*, with particular emphasis given to the relationship between rickettsial viability and lipoprotein purification.

## MATERIALS AND METHODS

*Rickettsial strain and working stock.* The AD strain of *C. burnetii* was employed. The working stock was prepared by harvesting the entire egg contents, minus shell, from embryos dying on the seventh, eighth, and ninth day postinoculation with *C. burnetii*. The harvest was suspended in one-third its volume of distilled water, homogenized in a Waring Blendor for 2 min, and centrifuged in a Sharples centrifuge (laboratory model) operated at 30,000 rev/min with a flow rate of 500 ml per min. The resulting supernatant fluid provided the stock suspension that was frozen and stored in a Dry Ice chest (−70 C).

*Assay procedure.* Total nitrogen determinations were made (Clark, 1943), by the semi micro-Kjeldahl method. Fat determinations were made by the chloroform extraction methods as described by the Association of Official Agricultural Chemists (1945). Total dry solids were determined with a Cenco moisture balance (Central Scientific Co., Chicago, Ill.).

Rickettsial infectivity was estimated by inoculating guinea pigs (250 to 400 g) intraperitoneally with 1-ml doses of serial, 10-fold dilutions made in Heart Infusion Broth (Difco). Daily rectal temperatures were obtained for each pig for 15 days after inoculation. At 21 days postinoculation, each guinea pig was bled by cardiac puncture; the blood was processed for serum, which was subsequently inactivated at 56 C for 30 min. Each

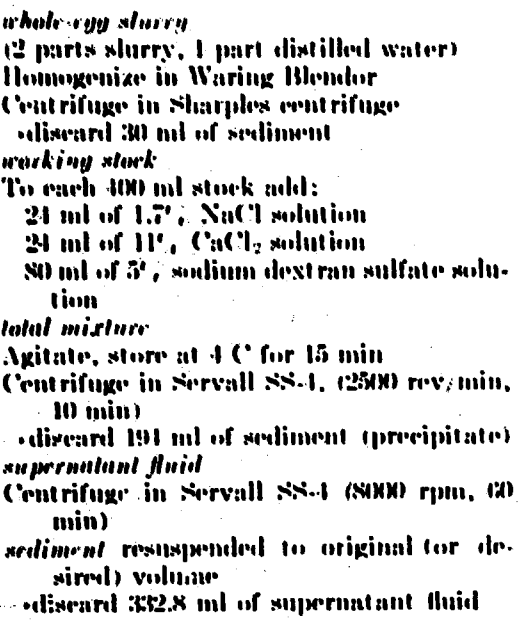


FIG. 1. *Process flow of dextran sulfate precipitation of whole-egg slurry. NaCl solution was from General Chemical Division, Allied Chemical and Dye Corp., New York, N.Y.; $CaCl_2$ solution was from Pharmacia Laboratories, Inc., Rochester, Minn.; sodium dextran sulfate solution was from J. T. Baker Chemical Co., Phillipsburg, N.J.*

inactivated serum was tested for the presence or absence of specific Q fever antibodies by the standard complement-fixation test. The criteria of infection were: (i) elevation of temperature of 40 C or greater for at least 2 consecutive days, and (ii) the presence of complement-fixing antibody to *C. burnetii* (in serum taken from inoculated animals). Data on infected pigs (any serum exhibiting a 4+ reaction at 1:4 dilution) for each inoculating dilution were totaled, and an end point was calculated by the method of Reed and Muench (1938). Infectivity data are expressed as $\log_{10}$ guinea pig intraperitoneal infectious doses per milliliter.

*Purification procedure.* Each test day, 400 ml of frozen, working stock were thawed and 24 ml of 1.7% NaCl plus 24 ml of 11% $CaCl_2$ were added to the stock and lightly agitated by hand, followed by the addition of 80 ml of 5% sodium dextran sulfate solution. A flocculent precipitate was separated by centrifugation (Servall SS-4; Ivan Sorvall, Inc., Norwalk, Conn.) at 2,500 rev/min for 10 min (755 × *g*). The supernatant fraction, designated as dextran-soluble lipoprotein, was decanted and stored at 4 C. The precipitate, designated as dextran sulfate-precipitable lipoprotein was discarded.

To further purify and concentrate the rickettsiae, the supernatant fluid was centrifuged in a refrigerated Servall centrifuge (SS-4) at 8,000 rev/min for 1 hr (7,710 × *g*). The rickettsial-rich sediment was recovered and resuspended in distilled water to the original volume of stock as shown in Fig. 1.

*Microscopic examination.* Specimens for microscopic examination were prepared by resuspending the final sediment in distilled water as a 1:20 dilution. Smears were stained by the method of Macchiavello (1937) and examined under the oil-immersion objective of a light microscope (Bausch & Lomb model 1-LM-4). Electron micrographs of the suspension were made by use of an RCA model EMU-2 instrument. The grids were shadowed with uranium, and pictures were made at an angle of 30° and a magnification of 20,400 times.

## RESULTS

Six representative experiments demonstrating the purification of *C. burnetii* are shown in Table 1. For comparison of the degree of purification, data from analyses of dry solids, protein nitrogen, and fat were obtained from the initial suspension (working stock), partially purified supernatant fluid, and the reconstituted sediment. The initial suspension contained approximately 17% total dry solids, 13,406 mg of nitrogen, and 71.63 mg of fat per ml. The partially purified suspension contained 4.46% total dry solids, 6,605 mg of nitrogen, and 1.06 mg of total fat per ml. The final purified suspension contained 0.05% total dry solids, 0.42 mg of nitrogen, and 0.22 mg of fat per ml. The partially purified suspension, when compared with the initial suspension, contained 78.05% less dry solids, 50.99% less total nitrogen, and 98.81% less fat; moreover, the final suspension showed a reduction of 99.73% total dry solids, 99.69% total nitrogen, and 99.84% fats. The final sediment, before resuspension with diluent, constituted approximately 0.3% of the volume of the original suspension or working stock.

Microscopic examination of smears made from the purified suspension and stained by the method of Macchiavello (1937) showed that this preparation consisted of relatively pure *C. burnetii*. An examination of the electron micrograph (Fig. 2) indicates that the rickettsiae are virtually free from the insoluble solids of the egg substrate.

## DISCUSSION

A method has been devised for preparing purified suspensions of viable *C. burnetii*, based on the centrifugation of dextran sulfate-precipitated egg suspensions, followed by another centrifugation to concentrate the rickettsiae from the partially purified suspension. In contrast to previous precipitation methods, particularly the use of nonaqueous organic solvents, it was possi-

TABLE 1. *Six representative experiments demonstrating the purification of Coxiella burnetii*

| Expt | *C. burnetii* suspension* | Concn of *C. burnetii* (log guinea pig intrap. mlt) | Vol | Total dry solids | Total nitrogen | Total fat | Degree of purification: Contents of dry solids removed from initial suspension | Degree of purification: Contents of nitrogen removed from initial suspension | Degree of purification: Contents of fat removed from initial suspension |
|---|---|---|---|---|---|---|---|---|---|
| | | | *ml* | % | *mg/ml* | *mg/ml* | % | % | % |
| I | Initial | $2.6 \times 10^{10}$ | 400 | 17.0 | 14.133 | 68.40 | | | |
| | Supernatant | $1.2 \times 10^{10}$ | 334 | 4.3 | 6.702 | 3.70 | 78.90 | 52.58 | 95.40 |
| | Final | $1.5 \times 10^{10}$ | 400 | 0.030 | 0.044 | 0.030 | 99.71 | 99.69 | 99.96 |
| II | Initial | $3.1 \times 10^{10}$ | 400 | 17.0 | 13.398 | 65.50 | | | |
| | Supernatant | $2.7 \times 10^{10}$ | 344 | 4.7 | 6.516 | 0.01 | 76.22 | 51.17 | 99.99 |
| | Final | $2.5 \times 10^{10}$ | 400 | 0.050 | 0.042 | 0.02 | 99.71 | 99.69 | 99.99 |
| III | Initial | $4 \times 10^{10}$ | 400 | 17.0 | 13.342 | 65.50 | | | |
| | Supernatant | $3.5 \times 10^{10}$ | 328 | 4.2 | 6.643 | 0.80 | 79.74 | 50.21 | 97.79 |
| | Final | $3.7 \times 10^{10}$ | 400 | 0.050 | 0.030 | 1.20 | 99.71 | 99.78 | 98.17 |
| IV | Initial | $7.0 \times 10^{10}$ | 400 | 17.2 | 13.369 | 66.80 | | | |
| | Supernatant | $4.5 \times 10^{10}$ | 328 | 4.7 | 6.632 | 0.96 | 77.32 | 51.40 | 99.77 |
| | Final | $5.5 \times 10^{10}$ | 400 | 0.06 | 0.032 | 0.04 | 99.76 | 99.77 | 99.95 |
| V | Initial | $1 \times 10^{10}$ | 400 | 17.0 | 12.835 | 81.80 | | | |
| | Supernatant | $3 \times 10^{10}$ | 334 | 4.6 | 6.765 | 0.08 | 77.40 | 47.30 | 99.91 |
| | Final | $3.5 \times 10^{10}$ | 400 | 0.04 | 0.073 | 0.02 | 99.77 | 99.43 | 99.98 |
| VI | Initial | $9 \times 10^{10}$ | 400 | 17.0 | 13.362 | 80.80 | | | |
| | Supernatant | $4.5 \times 10^{10}$ | 336 | 4.3 | 6.373 | 0.80 | 78.75 | 52.31 | 99.01 |
| | Final | $6.0 \times 10^{10}$ | 400 | 0.05 | 0.035 | 0.03 | 99.71 | 99.76 | 99.97 |

* Initial = initial suspension, 66% whole egg; supernatant = partially purified supernatant fluid; final = final purified suspension.

ble to preferentially separate the organism from the lipoprotein or lipidlike solids without a substantial loss in viability.

To establish an optimal level of precipitation, that is, maximal removal of extraneous solids with minimal rickettsial loss, a series of preliminary titrations, based on the work of Sakagami and Zilversmit (1962), were made with solutions containing different concentrations of dextran sulfate, calcium chloride, and sodium chloride. Slight modification of their procedure was necessary to obtain the optimal concentration of chemicals required for maximal purification; purification was measured quickly by the amount of dry solids removed from the supernatant fluid. The concentration of each chemical additive is dependent upon the level of solids (total dry) available in the egg suspension. Reducing the solids level of the egg suspension by the addition of various quantities of distilled water changes the requirement for the chemical. For example, maximal precipitation for the initial or working stock suspension containing 8.5% total dry solids can be achieved with half the concentration of chemicals required for a working stock containing 17% dry solids.

According to Ribi and Hoyer (1960), the density of *C. burnetii* is 1.11 to 1.18. The success of this method might be due to the preferential precipitation of the low-density lipoproteins (1.063 or less) at a neutral *p*H, leaving the

FIG. 2. *Electron micrograph of Coxiella burnetii 20,400* ×.

rickettsiae in the supernatant fraction (high density fraction) which has a density range of 1.063 to 1.21.

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